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Use of whole genome deep sequencing to define emerging minority variants in virus envelope genes in herpesvirus treated with novel antimicrobial K21

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ABSTRACT

New antivirals are required to prevent rising antimicrobial resistance from replication inhibitors. The aim of this study was to analyse the range of emerging mutations in herpesvirus by whole genome deep sequencing. We tested human herpesvirus 6 treatment with novel antiviral K21, where evidence indicated distinct effects on virus envelope proteins. We treated BACmid cloned virus in order to analyse mechanisms and candidate targets for resistance. Illumina based next generation sequencing technology enabled analyses of mutations in 85 genes to depths of 10,000 per base detecting low prevalent minority variants (<1%). After four passages in tissue culture the untreated virus accumulated mutations in infected cells giving an emerging mixed population (45–73%) of non-synonymous SNPs in six genes including two envelope glycoproteins. Strikingly, treatment with K21 did not accumulate the passage mutations; instead a high frequency mutation was selected in envelope protein gQ2, part of the gH/gL complex essential for herpesvirus infection. This introduced a stop codon encoding a truncation mutation previously observed in increased virion production. There was reduced detection of the glycoprotein complex in infected cells. This supports a novel pathway for K21 targeting virion envelopes distinct from replication inhibition.

K21 is a recently described quaternary ammonium silane molecule representing a new class of drug with antimicrobial and antiviral properties. Quaternary ammonium compounds can solubilise phospholipid bilayers leading to cell lysis and can affect virus envelopes (Gong et al., 2012; Tsao et al., 1989; Tuladhar et al., 2012). A recent clinical trial showed bacterial contact killing and reduced biofilms in inserts placed in the oral cavity for applications to dental healthcare (Liu et al., 2016). Herpesvirus also exist in the oral cavity and *in vitro* treatment with K21 showed antiviral effects with log reductions in herpes simplex virus type 1, HSV-1, and human herpesvirus 6A, HHV-6A (Gulve et al., 2016). The mechanism of action was not defined, although there was an effect on envelope glycoprotein gB expression, which could be from reduced virus cellular entry or signalling. We used HHV-6A as a model to analyse the mechanism of action of K21 through characterisation of potential resistance mutations utilising whole-genome deep-sequencing technologies we developed for this virus (Tweedy et al., 2015b, 2016).

HHV-6A is Roseolovirus betaherpesvirus, and linked with neurological and cardiac disorders in immune suppressed or naive patients (Gompels, 2016), including fatal infant myocarditis (Simpson et al., 2016; Stefanski et al., 2016). Moreover, Roseoloviruses are unique among human herpesviruses in integrating their genome in the human germline at the chromosomal telomere, termed ciHHV-6A and ciHHV-6B. This affects approximately 1% of people worldwide – upwards of 70 million people at risk of virus reactivation in every cell, with evidence for links to cardiac disease (Gravel et al., 2015; Kuhl et al., 2015; Tweedy et al., 2015b, 2016). Current drug treatment for herpesviruses include acyclovir for HSV and off licence use of valganciclovir for HHV-6A. These drug classes affect virus DNA replication and are prone to antimicrobial resistance mutations, new treatment options and methods to analyse their efficacy are required.

We previously developed methodologies using target enrichment with next generation sequencing to characterise specifically

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the virus sequences separated from the human genome in order to characterise their differences and also applicable to direct testing of clinical materials. Combining this method with whole-genome deep-sequencing, we were able to significantly detected 'minor variants' down to 1% in a mixture and applied this technology to identify HHV-6A virus superinfection in patients with integrated virus genomes showing 1–30% mixtures (Tweedy et al., 2015b, 2016). Here we applied this methodology to characterise minor variants to provide an insight into the mechanism of drug selection using K21, as a new drug class targeting the virus envelope.

HHV-6A strain U1102 was grown in JJhan cells and BAC cloned HHV-6A U1102 (BAC virus) (kindly provided by Y. Mori, Kobe University, Japan) (Tang et al., 2010, 2011) in HSB2 cells. BAC viruses were passaged four times as identically in the presence or absence of K21 drug (0.13 μM) respectively, as previously described (Gulve et al., 2016) and as below. Equivalent total infected cell DNA samples were extracted (Qiagen) then prepared for target enrichment amplification and deep sequencing as we described (Tweedy et al., 2015a, 2016). Reference HHV-6A U1102 and ciHHV-6A strains (Tweedy et al., 2016, Table 1) were simultaneously re-sequenced to verify any identified SNPs (not shown). For the specific amplification, 36 primer pairs were used which amplified overlapping PCR products across the complete genome, which were purified, pooled and quantified using Qubit as described (Tweedy et al., 2015a, 2016). Next, Covaris sonicated DNA libraries were prepared, purified and random PCR amplified using adaptor tags (NEBNext DNA library kit). Tagged samples were run on an Illumina MiSeq and raw FASTQ files analysed after quality assessment and removal of adaptor tags and primer sequences as described (Tweedy et al., 2016). FASTQ reads were trimmed using Trimmomatic with quality scores applied, then assembled using Samtools and BWA-mem by mapping to reference HHV-6A U1102 reference genome (HHV-6 U1102 NC_001664 and updated from *denovo* assemblies). Coverage and qualities were assessed, then contigs ordered with manual adjustment using Artemis, and RATT used to transfer annotations from the reference genome (Otto et al., 2011). SNP calling used both GATK UnifiedGenotyper and SAMtools mpileup, BCFtools, vcfutils varFilter pipeline, with quality scores >25 plus. SNPs were defined using a cut-off of minimum read depths of 10 as described (Phelan et al., 2016; Tweedy et al., 2015b, 2016). SNP databases were compiled and compared using Excel and custom Python scripts. To analyse K21 treatment effects, distinct SNPs compared between untreated and treated genomes were tabulated (Table 1). Average genome coverages were between 2000 and 10,000 read depths with a sensitivity SNP cut-off of 1%. Duplicate library compilations were >99% identical. Genome coverage from K21 treated compared to untreated samples were five-fold lower reflecting reduction of virus titre from the infected cells.

In the untreated BAC virus sample, there were 12 SNPs identified including two indels (one in the U86 IE2 repeat and one in a non-coding region of U100 between gQ1 and 2). These represented emerging populations with non-synonymous SNPs comprising 45%–73% of the sample compared to wild type. Six were non-synonymous changes in coding genes for U86 IE2, structural components U31 tegument, U33capsid, U50 capsid, and virion envelope glycoproteins U39 envelope glycoprotein gB (Thr193:Ala) and U100 gQ2 (Ser42:Leu), with only the U50 capsid and IE2 SNPs previously reported in other strains (Table 1). In contrast, comparisons between genomes of BAC virus treated and untreated showed the effects of K21 did not accumulate the population of non-synonymous variants in the BACmid population and retained the wild type U1102 sequence except for two coding changes (Table 1). Aside from three new non-coding/non miRNA SNPs, these were reduced from six coding changes to only two, U86 IE2 (an indel repeat Serine codon) and a second mutation in U100 gQ2, which introduced a stop codon giving a truncated gQ2 (Trp186:stop).

To examine gQ expression, the passaged BAC-U1102 and K21-BAC-U1102 viruses were lysed in lysis buffer, as described (Gulve et al., 2016) and in Fig. 1 then incubated with monoclonal antibody Ag gQ1 (a gift from Y. Mori, Kobe University, Japan) which immunoprecipitates the gH/gL/gQ1/gQ2 complex (Akkapaiboon et al., 2004; Tang et al., 2011). The antibody-complexes were bound using sepharose protein A, then eluted, separated on SDS-PAGE, then blotted onto nitrocellulose membranes. The separated

Table 1
Coding and non-coding SNPs in BAC HHV-6A U1102 with and without K21 treatment.

ORF[a]	RefSeq bp	RefSeq:SNP	Coding changes Codon or (reverse complement)	BAC virus	BAC virus K21	Comment, citation, strains
DR	8016	A:C		87%	–	End T2
DR	8080	G:GAC		86%	–	In ciHHV-6A 2284, 5055, 5814 KT895199.1 (Tweedy et al., 2016)
U left repeats	8561	AAC:A		100%	–	In HHV-6A (AJ AACAAC:A) KP257584 (Tweedy et al., 2015a)
U3	10116	A:AT		–	44%	Spliced, non-coding, polyA
U17	26147	A:T		–	99%	Spliced, non-coding
U19	28371	C:CA		52%	–	HHV-6A AJ, UTR
U31 tegument	45667	A:T	Asn314:Ile AAC:ATC	73%	–	
U33 capsid	52148	A:G	Tyr330:His (TAT:CAT)	54%	–	
U39 gB	61162	T:C	Thr193:Ala (ACG:GCG)	58%	–	
U50 capsid	81583	G:A	Ala258:Thr (GCA:ACA)	59%	–	In HHV-6A GS KC465951.1
U86 IE2	127092	C:CTGA	Ser976:SerSer (repeats ×9: ×10)	45%	65%	In CiHHV-6A 5055, 5814; (Tweedy et al., 2016)
U89	132303	TG:T		64%	79%	Non-coding outside exon
Kpn repeat	140872	G:A		96%	–	
U100 gQ2	146729	C:T	truncates gQ2; Trp186:stop (TGG:TGA)	–	100%	In 7/8 gQ2 cDNA clones (Tang et al., 2011)
U100	147793	C:T		–	99%	Non-coding between gQ1, gQ2
U100 gQ2	146862	G:A	Ser42:Leu (TCG:TTG)	60%	–	Not in gQ2 in Akkapaiboon et al. (2004)

[a] Coding changes in bold; shaded ORFs show only coding changes with K21 treatment.

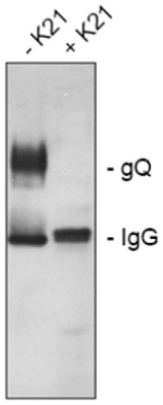

Fig. 1. Effect of K21 (0.13 μM) on glycoprotein gQ expression. As HHV-6A is a highly cell associated virus, HHV-6A infected HSB2 cells (cell associated virus and cell-free virus in culture supernatant) were combined with uninfected cells, at a ratio of 1:5, passaged four times in the presence of K21 or solvent control. Cells were cultured in RPMI 1640 media containing 10% FCS, 2 ng/ml of PHA and 5 pg/ml of IL-2. Two different sets of infections were carried out in parallel, one with K21 and the other with the solvent control. Once cytopathic effect was observed in the majority of cells (5–7 days post infection), cells were mixed once again with uninfected HSB2 cells at a ratio of 1:5. This step was repeated 4 times. After four infection cycles, total genomic DNA was isolated from infected cells for the further sequencing analysis as shown in Table 1, while protein lysates were also prepared for immunoprecipitation and immunoblotting studies shown here. Infected cell protein lysates were prepared from K21 treated or untreated cells and gQ complex expression analysed by immunoprecipitation and immunoblot showing the gQ complex (approximately 80 Kd).

proteins were then exposed to the same antibody, followed by washing, incubation with anti-mouse antibody conjugate, further washing and final film exposure as described (Gulve et al., 2016). Results showed abundant gQ complex detection in the untreated infected cells reduced to almost undetectable or degraded levels after K21 treatment (Fig. 1).

BAC viruses were used to investigate drug selection as the BACmid 'clones' the virus genome from mixtures of these cell-associated viruses using a bacterial artificial chromosome sequence, BAC, with plasmid derived sequences. However, the reconstituted virus has to replicate in cell culture and studies from another related herpesvirus, human cytomegalovirus, HCMV, show that complex mixtures of SNPs can accumulate during selection for efficient replication in tissue culture (Murrell et al., 2016). These included coding changes in components of the HCMV gH/gL pentameric complex with UL128/130/131 proteins. HHV-6A does not encode these components, but interestingly, the positional homologues are gQ1/gQ2 in the gH/gL/gQ1/gQ2 complex which were targeted in both the parent HHV-6A BAC virus and as second mutation in the K21 treated BAC virus.

As cited above, we applied K21 to virus stocks of infected cell preparations rather than purified cell free virions (Gulve et al., 2016) and Fig. 1. This mimics infection in vivo and these stocks have the highest virus titers compared to cell free virions, since the virus is highly cell associated. However, since K21 can disrupt membranes, most likely via its long alkyl side chains (Gulve et al., 2016; Tsao et al., 1989; Tuladhar et al., 2012), this may affect immature, defective or mutant virus in the infected cell preparation greater than mature released wild type virions. Although, the concentration of K21 used, 0.13 μM (Fig. 1), was 10 times lower than that determined for cellular toxicity, IC60 1.35 μM for HSB2 cells, (Gulve et al., 2016), different lipid and cholesterol compositions, including lipid rafts in the membranes of the infected cell compared to virus envelope (Tang et al., 2008), may affect membrane fluidity and sensitivity to K21. The K21 associated SNP is in gQ2 (Table 1), affects the gQ complex stability (Fig. 1), and gQ has been shown to interact with lipid rafts (Tang et al., 2008). Furthermore, SNPs may accumulate in passaged infected cells, as continual serial passage of virus-infected cells facilitates replication of defective particles. These would not be viable with reduced titers, removing sufficient helper virus required to propagate the defective particles. This may explain the striking effects from K21 treated BAC-U1102. This virus did not accumulate the culture passage non-synonymous minor variants, with the exception of only two coding SNPs. One was already present in the BAC virus, the U86 SNP a repeat in a known coding exon (reverse complement TCA Serine insertion 9–10×), which adds a further serine also present in other strains and unlikely to account for the differences in envelope glycoprotein expression.

There were three new SNPs identified after K21 treatment of the BAC virus, which could indicate direct protein targets in addition to overall possible saponic effects on the membrane. However, two of these, U3 and U17, were outside known coding regions or functional micro RNAs (Nukui et al., 2015). In contrast, the third SNP was a second mutation of the spliced U100 gene encoding a truncated gQ2 glycoprotein, part of the gH/gL/gQ1/gQ2 complex. This glycoprotein complex is essential for cellular infection, mediates cellular fusion and gQ2 deletion disrupts infection (Tang et al., 2011). The complex interacts with a receptor, the CD46 molecule (Hansen et al., 2017; Jasirwan et al., 2014; Mori et al., 2003; Santoro et al., 1999, 2003). Interestingly, this truncated gQ2 SNP has been previously described as a polymorphism in the passaged HHV-6A U1102 stocks used to derive the BACmid of strain U1102 (clone G-1) as applied here. It was observed that 7/8 cDNAs from those virus stocks contained this polymorphism (Tang et al., 2011). However, our analyses of both the parental isolate HHV-6 U1102 and the reconstituted BAC-HHV-6A U1102 did not show this SNP, at 1% sensitivity. The passaged BAC virus reverted to wild type. However, markedly after K21 treatment, 100% of the resultant virus stock regained this gQ2 SNP.

The expression of gQ2 in the K21 treated virus was compared to the untreated virus. The results showed no detection of the gQ complex as detected by immunoprecipitation with a gQ1 specific monoclonal antibody. This antibody immunoprecipitates the gH/gL/gQ1/gQ2 glycoprotein complex, but is specific for gQ1 in western blots. Previous experiments with these K21 treated stocks showed overall 63% decreased virus titers, but similar expression of replication protein p41 (Gulve et al., 2016). While glycoprotein gQ was reduced as shown here. This suggests the SNP causing truncation of gQ2 may destabilise the gQ complex or alter its signalling. Previous studies on this truncated gQ2 show it can form the gQ complex in transfected cells, and maintains interaction with receptor CD46 (Tang et al., 2011). However, interestingly, studies on expression of the truncated gQ2 in virus infected cells, also shows instability in infected cells compared to virions (Tang et al., 2011). Possibly this effect of enhanced stability in virions increases the potential resistance to K21.

It was surprising that the BAC virus parent accumulated prominent mutation mixtures. The original HHV-6A strains were isolated on cord blood stem cells. While T leukemic cell lines JJhan or HSB2 were used to passage reconstituted BAC virus and this may affect SNP accumulation. Therefore, characterisation of host interactions, genome integration, immune interactions, and antiviral resistance may need to take account of the effects of possible diverse mixed genome populations in BAC passaged virus. The BAC virus have SNPs affecting infection similar to the effects on related HCMV, which also accumulate mutations affecting the gH/gL complex sensitive to cell tropism. New BACs may be required to analyse antivirals or different culturing protocols. In HCMV BAC virus, further conditional mutations are required to maintain the wild type genome (Murrell et al., 2016). However, results here

showed novel antiviral, K21 treatment did not accumulate the passage-associated HHV-6A BAC SNPS; the K21 virus retained the wild type reference strain except for the gQ2 truncation. It would be of interest to test HCMV effects, to target salivary transmission of both viruses.

Additional study of these mutations from BAC and K21 treated virus and new clinical isolates would provide further understanding of virus infection and antimicrobial mechanisms. K21, as a new class of antiviral targeting virus envelope and infection, may have promise in combinations with antivirals targeting replication.

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Conflict of interest

None declared.

Author contributions

UAG and JT designed the study; BKP performed virus and protein experiments; JT performed sequencing experiments; UAG, BKP and JT analysed the data and contributed to manuscript preparation; UAG wrote the final manuscript.

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